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Dissection of the NF-Y transcriptional activation potential

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ABSTRACT

NF-Y is a trimeric CCAAT-binding factor with histone fold subunits (NF-YB/NF-YC) and bipartite activation domains located on NF-YA and NF-YC. We reconstituted the NF-Y activation potential *in vivo* with GAL4 DBD fusions. In the GAL4-YA configuration, activation requires co-expression of the three subunits; with GAL4-YB and GAL4-YC, transfections of the histone fold partners are sufficient, provided that the Q-rich domain of NF-YC is present. Combinations of mutants indicate that the Q-rich domains of NF-YA and NF-YC are redundant in the trimeric complex. Glutamines 101 and 102 of NF-YA are required for activity. We assayed NF-Y on different promoter targets, containing single or multiple GAL4 sites: whereas on a single site NF-Y is nearly as powerful as VP16, on multiple sites neither synergistic nor additive effects are observed. NF-Y activates TATA and Inr core elements and the overall potency is in the same range as other Q-rich and Pro-rich activation domains. These results represent the first *in vivo* evidence of subunit interactions studies and further support the hypothesis that NF-Y is a general promoter organizer rather than a brute activator.

INTRODUCTION

Regulation of gene expression is dictated by DNA regulatory elements found in the proximity of (promoters) and at a distance from (enhancers) transcribed genes. The core promoter elements (TATA and Initiator) are targets of the general transcription machinery, being involved in the activation of all Pol II transcribed genes. In contrast, upstream sequences are a combinatorial puzzle of specific elements that vary among promoters and are recognized by proteins binding DNA with high specificity. In addition to DNA binding, these proteins usually possess a transcriptional activation function. Originally defined in transfection assays, the activation domains recognize targets in the general transcription apparatus (or holoenzyme). Molecular dissection of different activators led to the discovery of disparate protein stretches: Q-rich, acidic, Pro-rich, Ile-rich etc. (for a review see 1).

Among upstream elements, the CCAAT box is one of the most common, being found in 25% of eukaryotic promoters (2,3). This box is essentially recognized by NF-Y (also termed CBF), a ubiquitous trimeric protein composed of NF-YA (CBF-B), NF-YB (CBF-A) and NF-YC (CBF-C), all necessary for subunit association and CCAAT binding (for reviews see 3,4). NF-Y genes have been identified in many eukaryotic species: the yeast HAP2/3/5 complex, for example, has very similar structural features and is involved in the activation of cytocrome genes (5 and references therein). All three subunits share a conserved domain that is nearly identical across evolution. NF-YA associates with a pre-formed NF-YB/NF-YC dimer; NF-YB and NF-YC dimerize tightly via their histone fold motifs (HFM), a 65 amino acid stretch common to all histones that is required for nucleosome formation (6,7). The HFM allows histones to dimerize with companion subunits and interact with DNA. The HFM is shared with low sequence identity (10–18%) by other proteins involved in transcriptional regulation: TBP-associated factors $hTAF_{II}80$, $hTAF_{II}31$, $hTAF_{II}28$, $hTAF_{II}18$ and $hTAF_{II}20$ (8) and the two subunits of the TBP-binding protein NC2 (also called Dr1/DRAP1), a global repressor of basal transcription (9). The NF-Y trimer has high affinity and specificity for CCAAT boxes (3,4) and recent biochemical analysis suggests that NF-Y bends and twists DNA with angles that are highly reminiscent of nucleosomal structures (10,11).

NF-Y has two separate activation domains, as defined by studies with recombinant proteins *in vitro* and GAL4 constructs on isolated subunits *in vivo* (12–15). They are on NF-YA and NF-YC and both are alike, namely rich in glutamines and hydrophobic amino acids. In contrast, NF-YB seems devoid of activating potential (15). Since the search for an NF-Y-free cell line in which to test NF-Y activity with no endogenous background has been in vain so far, we pursued our analysis of NF-Y activation by recapitulating it with GAL4 fusions on a number of different targets and compared it with different classes of transcriptional activation domains.

MATERIALS AND METHODS

Plasmid constructions

G4-YA, G4-YA9, G4-YA12, G4-YB and G4-YB4 were described previously (12,15). The NF-YA expression vector was generated by excising the GAL4 DBD (DNA-binding domain) from G4-YA13 (15) cutting with *Hin*dIII and *Cla*I, fill-in with Klenow fragment and religation. Similarly, the NF-YB was obtained from G4-YB by cutting with *Eco*RI and *Cla*I, fill-in and religation. The human NF-YC overlapping clones

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described in Bellorini *et al*. (16) were used to generate the full-length NF-YC fragment by PCR, which was inserted into pGAL4Poly (17) between the *Kpn*I and *Bam*HI sites, to generate G4-YC. This plasmid was used to obtain the NF-YC vector, excising the GAL4 DBD by digestion with *Eco*RI and *Xho*I and religation. The NF-YCN mutant was derived by cutting NF-YC with *Cla*I and *Bam*HI, fill-in with Klenow fragment and religation: this eliminates amino acids 116–335 of NF-YC, containing the C-terminus of the protein. The Qm and Im mutations were generated with primers containing suitable sequences that substituted Q101–Q102 and I102–I104 of NF-YA with alanines; the resulting PCR fragments were cut with *Pst*I and exchanged for the wild-type fragment in the G4-YA12 and NF-YA plasmids.

The reporter plasmids were as described previously (17–19). The G4-SP1, G4-OCT1, G4-OCT2 and G4-AP2 plasmids were as described in Kunzler *et al*. (20) and were a kind gift of Dr W. Schaffner (University of Zurich, Zurich, Switzerland).

Cell transfections

Mouse NIH 3T3 fibroblasts were maintained in Dulbecco's minimum essential medium, supplemented with 10% fetal calf serum. Cells were co-transfected with 1 µg of activating plasmids (except in Fig. 2A–C), 2 µg of the plasmids containing the luciferase or CAT reporter genes, 3 µg of pNβGal plasmid (a gift of S. Weisz, University of Naples, Naples, Italy) for control of transfection efficiency. The total amount of transfected DNA was kept constant (15 µg) with plasmid pBluescript. All plasmids were purified by centrifugation using cesium chloride gradients. Cells were transfected by the standard calcium phosphate method, recovered 48 h after transfection, washed in phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) and resuspended in Reporter Assay Reagent (Promega). Luciferase activity was measured according to Serra *et al*. (15). β-Galactosidase activity was measured in 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 50 mM 2-mercaptoethanol and 0.66 mg/ml *O*-nitrophenyl-β-D-galactopyranoside (ONPG) in 500 µl final volume. After 30 min incubation at 37°C, the reaction was stopped by adding 1 ml of 1 M Na_2CO_3 and the A_{420nm} was measured. Luciferase activity values were corrected using as a reference the β-galactosidase activity expressed in each sample. CAT assays were carried out by the sensitive quantitative phase extraction method as previously described (21). Results were normalized according to the β-galactosidase actvity. A minimum of two independent transfections in duplicate were done; most of the values are based on 6–10 transfections.

Western blots were performed as described (15), using an anti-GAL4 monoclonal antibody kindly provided by Y. Lutz (IGBMC, Strasbourg, France).

RESULTS

Recapitulation of NF-Y activation

We have previously shown that constructs containing a wild-type NF-YA, either the long or short form (12), fused to the 1–147 DBD of GAL4 are unable to activate transcription from a promoter containing two GAL4 sites and a TATA box, while similar constructs devoid of the evolutionarily conserved HAP2 homology domain, but containing the Q-rich and S/T-rich regions, are active in a number of cell lines (15). Using western blot analysis, all NF-YA proteins were shown to be equally well expressed in eukaryotic cells, ruling out trivial explanations for this phenomenon, such as lack of expression or protein degradation (15). Two possible mechanisms can be envisaged: (i) the limited amount of endogenous NF-YB/NF-YC dimer in the transfected cells is unable to assist GAL4 NF-YA in this activation system; (ii) a self-squelching mechanism, due to overexpression of the activator plasmid, often encountered in these assays. The first hypothesis was checked by co-transfecting the G4-YA expression plasmid together with NF-YB and NF-YC expression vectors under the control of the efficient SV40 enhancer (Fig. 1). The readout was a plasmid containing five GAL4 binding sites in front of a TATA box (G5-TATA) driving the luciferase reporter gene (18); transfections were carried out in mouse NIH 3T3 fibroblasts. Figure 2A shows the results of several such experiments normalized with an internal β-Gal plasmid for transfection efficiency. As previously noted on two GAL4 sites, the G4-YA construct gave no activation, even below that of the GAL4 1–147 negative control (see below). Addition of either NF-YB or NF-YC singularly had no influence

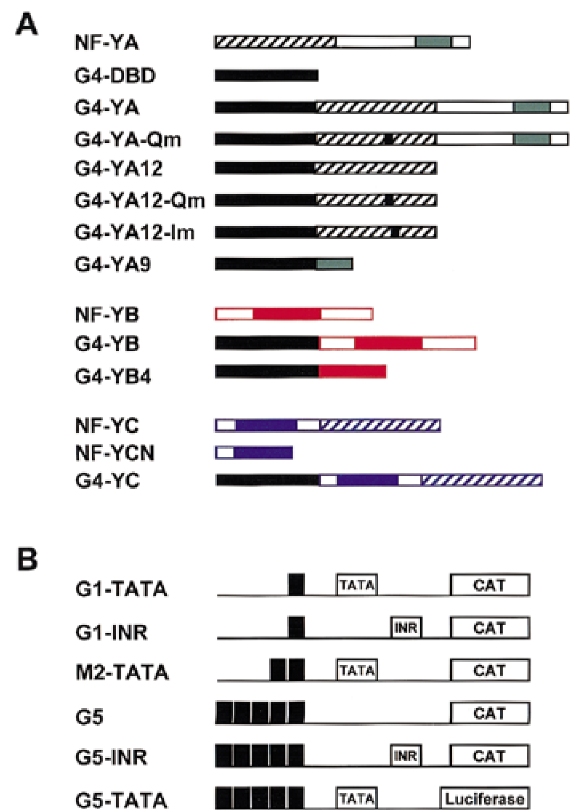


Figure 1. Vectors used in the transfection studies. (**A**) The G4-YA and G4-YB fusion vectors were as described before (12,15) and contain amino acids 1–147 of GAL4. The G4-YC vector contains wild-type NF-YC fused to the same domain. The NF-YA, NF-YB and NF-YC vectors are derived from the GAL4 fusions by excision of the GAL4 DBD. Hatched boxes refer to the NF-YA and NF-YC Q-rich domains, filled boxes to the homology domains. (**B**) The reporter vectors were as described (15–17) and contain one, two or five GAL4 sites in front of TATA or Inr elements.

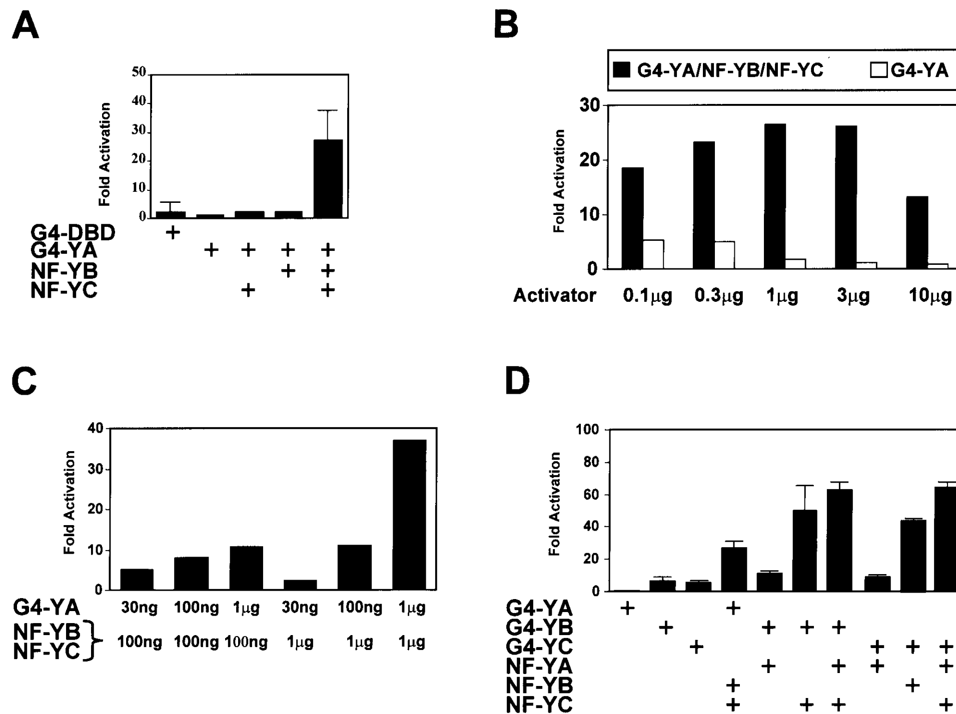


Figure 2. Transcriptional activation of the NF-Y trimer. (**A**) NIH 3T3 fibroblasts were transfected with 2 μg of the indicated activators and 2 μg of the G5-TATA reporter. Values indicate the fold activation over the G4-YA negative control. (**B**) Dose–response characteristics of G4-YA/NF-YB/NF-YC trimer (gray box) and of G4-YA (white box) on G5-TATA. (**C**) As above, except that the indicated amounts of activators (G4-YA and G4-YA/NF-YB/NF-YC) were transfected. (**D**) Different combinations of the expression vectors (1 μg each) were transfected. Values are calculated as fold activation over the G4-YA negative control.

on this low activity, while co-expression of all NF-Y subunits increased transcription almost 30-fold over the G4-YA background; no effect was seen with NF-YB/NF-YC, either alone or in combination, in the absence of G4-YA (not shown). Thus, reconstitution of the normal NF-Y trimeric interactions is required for transcriptional activation. To investigate whether the negative effect observed on G4-YA alone was due to a self-squelching mechanism, we performed dose–response studies of the G4-YA/NF-YB/NF-YC trimer with increasing concentrations of the three vectors (Fig. 2B) or by transfecting different amounts of G4-YA and NF-YB/NF-YC (Fig. 2C). Figure 2B indicates that although low activity was seen at low G4-YA concentrations (5-fold over the G4-DBD negative control) the trimer was clearly more active at all DNA concentrations, including at 10 μg, where a small negative effect was seen. Figure 2C shows that low levels (30 ng) of G4-YA do not activate further and that comparative amounts of each subunit were required to obtain maximal activation, visible at 100 ng and maximal at 1 μg. We therefore decided to use 1 μg of transfected plasmids for the subsequent experiments. Overall these experiments rule out that squelching is responsible for the lack of activity of G4-YA and favor the idea that the NF-YB/NF-YC dimer is indeed limiting.

We co-transfected G4-YB, previously shown to be largely inactive, with NF-YA and/or NF-YC and G4-YC with NF-YA and/or NF-YB. In these configurations, the GAL4 constructs gave essentially negative results when assayed alone (Fig. 2D): this is noteworthy for NF-YC, since it contains the Q-rich domain previously shown to be active in such an assay and with recombinant proteins *in vitro* (14): this result probably mirrors the lack of activity of G4-YA. Co-transfections with NF-YA were no more active, a result in line with the lack of interactions of this subunit with isolated NF-YB or NF-YC. Addition of all three subunits, as in G4-YA, activated transcription robustly. However, co-transfections of both histone fold subunits also gave strong activation (consider G4-YB/NF-YC and G4-YC/NF-YB). These results are the first indication that co-transfection of all three NF-Y subunits *in vivo* yields functional proteins and recapitulate the NF-Y activation potential, perfectly matching the NF-Y subunit interactions as determined by *in vitro* binding studies (4). The exceptions are the HFM dimers, which activate even in the in the absence of NF-YA.

Because of the apparent modest effect of the addition of NF-YA on GAL4 HFM dimers, we wished to further investigate the role of the Q-rich domains of NF-YA and NF-YC in this system: previous work showed that they are active with isolated GAL4 constructs, but not in the context of wild-type proteins (14,15; Fig. 2D). We co-transfected different combinations of mutants G4-YA9, G4-YB4, devoid of protein sequences outside the yeast homology domains, and NF-YCN, containing the HAP5 homology domain, but lacking the C-terminal Q-rich region. These experiments gave results consistent with the notion that the NF-YA and NF-YC activation domains are in the Q-rich domains (Fig. 3): co-transfections of NF-YB/NF-YC with G4-YA9 activated transcription better than with G4-YA, suggesting that NF-YA regions outside the HAP2 homology domain are

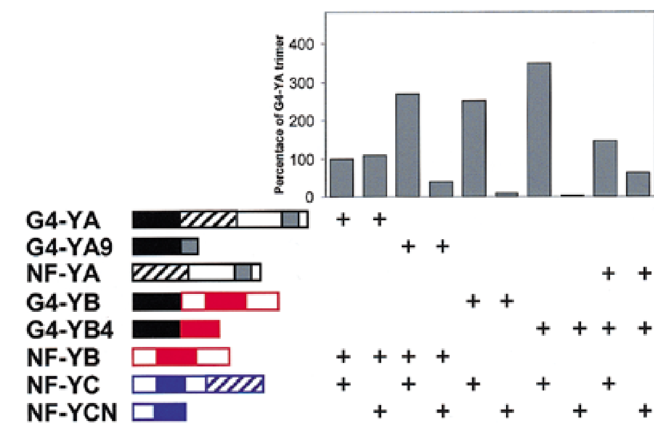


Figure 3. Transfections with NF-Y homology domain mutants. The indicated combinations (Fig. 1) of the NF-Y mutants were assayed on G5-TATA and the values obtained are plotted as a percentage of the activity of the G4-YA/NF-YB/NF-YC trimeric combination.

expendable and that NF-YC activation is sufficient. On the other hand, removal of the latter, as in NF-YCN, decreased activity considerably. Interestingly, activities of the HFM dimers were similar with G4-YB and G4-YB4, suggesting that NF-YB domains outside the HAP3 homology domain are neither required for activation nor for subunit interactions *in vivo*. Addition of NF-YA to HFM dimers lacking the NF-YC C-terminal domain restored activity almost completely (compare G4-YB4/NF-YCN with G4-YB4/NF-YCN/NF-YA). Note that low activities are not due to lack of expression or protein degradation, as checked in western blots (15; not shown). Taken together, these results are in line with the idea that one activation domain, either from NF-YA or NF-YC, is sufficient to give near maximal activity.

Mutations in the NF-YA activation doman

A resemblance between NF-YA and the NTF-1/CP2/LBP-1 transcription factor was detected at the level of the primary amino acid sequence, in a region rich in glutamines and isoleucines: the latter, but not the former, were found to be essential for NTF-1/CP2/LBP-1 activation (22). To ascertain whether this homology was significant for NF-YA and whether NF-YA is dependent on glutamine or isoleucine residues, we constructed two mutants in the region of homology with NTF-1: Q101 and Q102 were mutated to A101 and A102 and I103–I105 to A103–A105 (Fig. 4A). They were inserted in the transcription-competent G4-YA12 construct and co-transfected with the G5-TATA-luciferase reporter. Figure 4B shows the results of these experiments: the G4-YA12-Qm mutation essentially abolished function (compare G4-YA12 with G4-YA12-Qm), while G4-YA12-Im reduced it to 60%. To determine whether the effect of the mutations could be due to differential levels of protein expression we performed western blot analysis on extracts derived from the transfections with anti-GAL4 monoclonal antibodies (15): both G4-YA12-Qm and G4-YA12-Im showed levels that were actually higher than the wild-type G4-YA12 control, ruling out the possibility that lack of activation is due to lack of expression or degradation of the proteins (Fig. 4C). We also transferred the Qm mutaton in the G4-YA configuration:

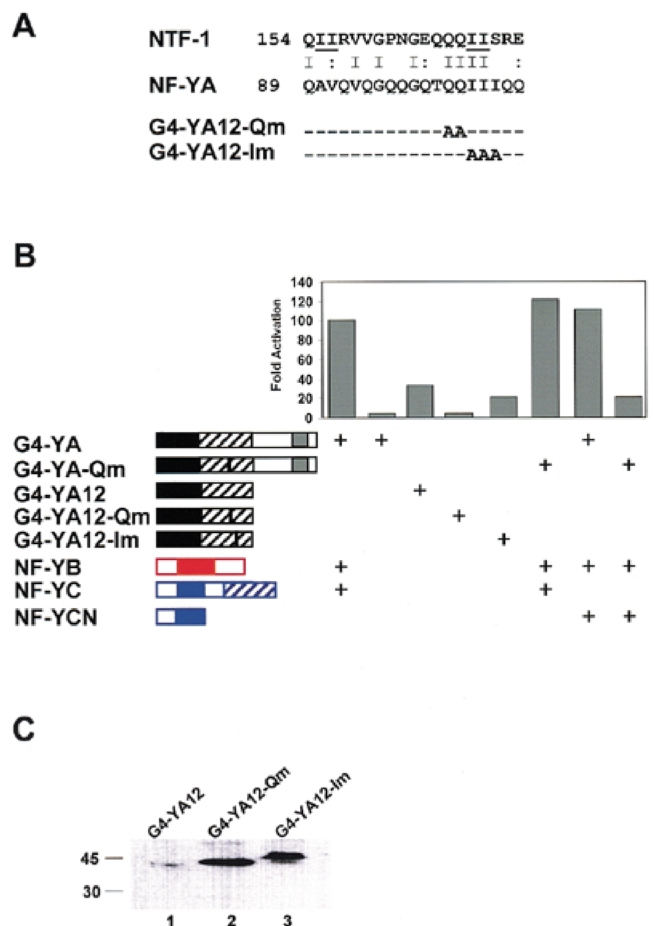


Figure 4. Analysis of NF-YA mutants in the activating region. (**A**) Sequences of the *Drosophila* NTF1 activator and of NF-YA are aligned (22). Underlined amino acids indicate residues that are important for NTF1 function in transfection experiments. The G4-YA12-Qm and G4-YA12-Im mutations are indicated. (**B**) Activation of the different NF-Y combinations on the G5-TATA reporter. Results are calculated as a percentage of the G4-YA/NF-YB/NF-YC combination; standard deviations were <20%. (**C**) Western blot analysis of extracts transfected with G4-YA12 (lane 1), G4-YA12-Qm (lane 2) and G4-YA12-Im (lane 3).

the effect of the alanine mutations is also evident (6-fold), but only if the C-terminus of NF-YC is absent from the trimer: if the two activation domains are present on the trimer, even this crippling mutation does not affect overall trimer function (compare G4-YA/NF-YB/NF-YC with G4-YA-Qm/NF-YB/NF-YC), whereas in the absence of the NF-YC C-domain, the effect of the Q mutations becomes apparent (compare G4-YA/NF-YB/NF-YCN and GA-YA-Qm/NF-YB/NF-YCN). These data suggest that glutamines 101 and 102 are important for function of the NF-YA activation domain and reinforce the notion that the NF-YA and NF-YC activating regions are redundant.

Comparison with other activators

After reconstituting the NF-Y activation function, we decided to compare it with that of other transcription factors. For direct comparison, we co-transfected G4-SP1, G4-OCT1, G4-OCT2, G4-AP2 (20) and GAL4-VP16, in parallel with the NF-Y

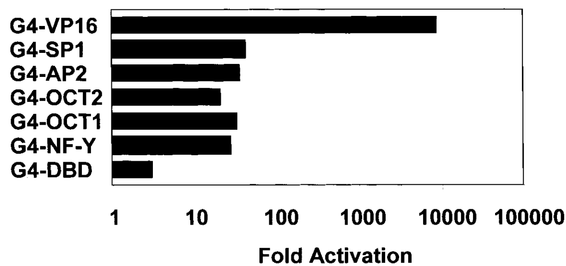
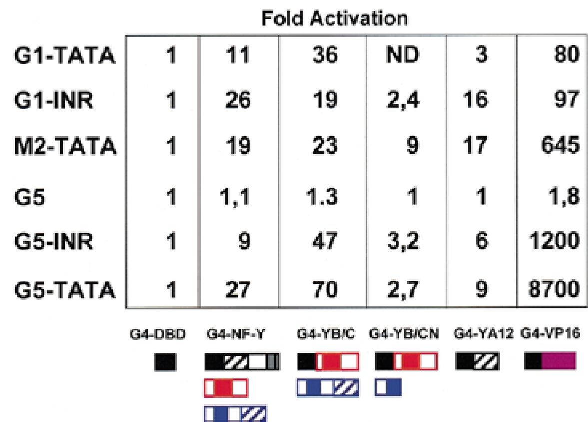


Figure 5. Comparison of NF-Y activation with other activators. Cells were transfected with 1 µg of the indicated activator (20), except for G4-VP16, for which 50 ng were used. The activity (on a logarithmic scale) is given as fold activation over the G4-YA negative control. Note that G4-DBD has a weak but reproducible activity. Standard deviations were <20% of the values.



Fold Activation

	G4-DBD	G4-NF-Y	G4-YB/C	G4-YB/CN	G4-YA12	G4-VP16
G1-TATA	1	11	36	ND	3	80
G1-INR	1	26	19	2,4	16	97
M2-TATA	1	19	23	9	17	645
G5	1	1,1	1.3	1	1	1,8
G5-INR	1	9	47	3,2	6	1200
G5-TATA	1	27	70	2,7	9	8700

Figure 6. NF-Y specificity on core promoter elements. The indicated targets decribed in Figure 1 were co-transfected with G4-DBD, G4-YA/NF-YB/NF-YC, G4-YB/NF-YC, G4-YA12 and G4-VP16. Normalized activities referred to G4-DBD as a negative control. Standard deviations were <25%.

trimer in the G4-YA configuration, on the same G5-TATA reporter. Results of these experiments are shown in Figure 5. As expected, the acidic G4-VP16 activated very efficiently (almost 10 000-fold); the Q-rich G4-SP1, G4-OCT1 and G4-OCT2 and the Pro-rich G4-AP2 also activated (25- to 40-fold on average); NF-Y trimer activation was clearly in the same range as these latter transcription factors. Thus, in our experimental conditions, NF-Y is not a powerful activator, such as those having an acidic domain, but belongs to the same class as other Q- and Pro-rich activators.

NF-Y activation of different promoter targets

Because of the wide promoter distribution of the CCAAT box, most often as a single site element, we tested the NF-Y activation potential on different promoter configurations; the targets used in this set of experiments are outlined in Figure 1 (17–19). When assayed on a promoter containing a single GAL4 site, the G4-YA/NF-YB/NF-YC trimer activated transcription 11-fold on TATA and 26-fold on Inr, over the baseline represented by the G4-DBD. The G4-YB/NF-YC dimer was comparable on Inr and more active (36-fold activation) on TATA. These activities were only slightly less than those of the powerful G4-VP16. On the M2-TATA promoter, which has two GAL4 sites, G4-VP16 activity increased considerably (645-fold activation), consistent with the synergistic effect described for this activator, while the activities of the trimer or the HFM dimer were comparable with those observed on G1-TATA and G1-INR. With five sites, with both TATA or Inr core elements, GAL4-VP16 activity further increased; the NF-Y trimer remained at similar levels (27-fold activation on TATA and only 9-fold on Inr); the G4-YB/NF-YC dimer was more active (70- and 47-fold the activity of the G4-DBD control). Note that removal of the C-terminus of NF-YC from the HFM dimer decreased activity to near background levels on all promoters. The isolated Q-rich region of G4-YA12 activated more weakly and synergy on multiple sites was also not evident (compare G1-INR and G5-INR). The activities of all these activators on a control promoter lacking both TATA and Inr elements (G5) were essentially basal. These results show that the NF-Y activation domains are equally efficient on different core promoter elements and, in contrast to VP16, display near maximal activity in the presence of a single GAL4 site.

DISCUSSION

In this study, we investigated the NF-Y activation potential with GAL4 constructs and reached the following major conclusions: NF-YA and NF-YC have separate activation domains; their overall activities are redundant in the trimer; NF-YA has crucial Q residues; the HFM subunits are sufficient to activate through the NF-YC C-terminus; NF-Y works on a single as well as on multiple sites; it activates through different core promoter elements.

One of the major problems in reconstituting binding and activation functions of this ubiquitous transcription factor *in vivo* is the lack of cell lines that provide an NF-Y-free background. For this reason, work on NF-Y activation focused on two reductionist approaches: *in vitro* transcription assays and transfections of isolated subunits on GAL4 constructs. These studies identified the N-terminus of NF-YA and C-terminus of NF-YC as large activation domains. The results presented here are in accordance with previous experiments, with one interesting exception: in the *in vitro* transcription experiments performed with recombinant NF-Y subunits on the α2(I) collagen promoter, the two Q-rich regions were not redundant, but contributed additively to activation (14). We find limited evidence for such an effect in our *in vivo* approach, namely the fact that the NF-Y trimer activates more (3-fold) than G4-YA12 and the small increase upon transfecting NF-YA with the HFM dimers. In contrast, other results from our study are at odds with this conclusion: (i) the HFM GAL4 contructs (lacking one Q-rich region) were consistently better than the trimer in the G4-YA configuration; (ii) removal of the NF-YC Q-rich region by deletion did not reduce activity of the trimers; (iii) removal of the NF-YA Q-rich region, either by deletion or mutation of important residues, if anything slightly increased activation. On the other hand, combinations lacking both the NF-YA and NF-YC activation domains were inactive. Since these phenomena are clearly not caused by lack of expression and are observed on promoters containing different core elements, they are unlikely to be due to a specific promoter architecture. We favor a different explanation for the lack of a clear additive effect of

the two activation domains in our assays. We have recently shown that the NF-YA and NF-YC Q-rich domains significantly influence bending angles and that they are both required for cooperative interactions among NF-Y molecules on twin CCAAT boxes, thus mediating protein–protein interactions (11); moreover, strict distance requirements between sites are necessary for cooperative interactions. It is in fact possible that the cooperative interactions between NF-Y and different transcription factors, described on several promoters (3,4), are also mediated by such domains. Moreover, binding of the NF-YA and NF-YC Q-rich domains to $dTAF_{II}110$ has been reported (23). Thus, NF-Y Q-rich regions might serve a dual function of stabilizing the binding of upstream factors and connecting them to core promoter elements. They probably evolved not only to provide a surface for direct binding to TAF_{II}s, but also as protein–protein interaction surfaces that increase the stability of DNA-binding complexes (11). Under these conditions, the full activation potential of NF-Y probably requires precise protein–protein interactions with nearby activators and might be underscored by the GAL4 assays, most likely oblivious of upstream interactions.

We have previously reported that splitting the long Q-rich region of NF-YA into two essentially abolishes activation of either half (15). NF-YA is found in two major forms, long and short NF-YA, that are the result of tissue-specific splicing of 28 amino acids in the Q-rich domain: both proteins, when assayed in activation assays, behaved essentially in the same way (12). It is therefore particularly noteworthy that mutations of two Q-rich domains have such a profound effect, especially since the mutagenesis was based on the observation by Attardi and Tjian that the activation domain of NTF1/LBP-1/CP2, which is rich in hydrophobic and Q residues, has homology with NF-YA (22). Since functional experiments in *Drosophila* established that isoleucines, but not glutamines, are important for NTF-1 activation (22), it was important to verify whether NF-YA belongs to the Q- rich or I-rich class of activators. One important conclusion of our study is the identification of two key glutamine residues at positions 101 and 102 of NF-YA, which are required for its function, while the nearby isoleucines are less essential. This result suggests that similar mutants can be derived from NF-YC, thus leading to the development of dominant-negative mutants for activation function.

Our data clearly indicate that even as a trimer, NF-Y is not among the most powerful of activators, but rather it is in the range of other Q-rich and Pro-rich activators (20,24–27). Since NF-Y has multiple activation domains, one should compare our data with those of a report showing that constructs containing multiple intramolecular VP16 activation domains on a single GAL4 DBD readily synergize among them (28).

It is particularly interesting to note the functional resemblance between NF-Y and SP1, as observed in activation assays. (i) The SP1-binding GC and the CCAAT boxes are the most frequent upstream promoter elements (2). (ii) One of the TFIID targets of SP1 Q-rich domains is $dTAF_{II}110$, required for SP1-mediated activation of trancription, which is also a possible target of NF-Y (23,29,30). (iii) Like NF-Y, SP1 functions well both on TATA and Inr core elements (31) and SP1 Q-rich domains fail to potentiate activation in *S.cerevisiae*, while functioning in *S.pombe*, which is closer to higher eukaryotes (20,32,33). Overall our data substantiate the hypothesis that their Q-rich domains serve similar functions, but differences also exist: GC boxes tend to be present in multiple copies, while the majority of CCAAT boxes are single and CCAAT multimerization brings no synergy to transcription (3). In DNA-binding and transcription experiments, cooperative binding of SP1 multimers is not observed, while synergistic activation is obtained when two or more SP1 sites are present on the promoter (34,35). This is due to multiple protein–protein interactions via different Q-rich domains (36): formation of higher order complexes, in the absence of DBDs are necessary for synergistic activation of SP1.

Phylogenetically, the *S.cerevisiae* HAP2/3/5 subunits have no Q-rich domain, but they associate with a fourth subunit (HAP4) containing a powerful acidic domain, required to render the CCAAT-binding complex transcriptionally active (5 and references therein); the HAP4 subunit has been apparently lost in other phyla, where the activating information has been incorporated within the core DNA-binding subunits: *Xenopus*, *Schistosoma mansoni* and sea urchin NF-YA have large Q-rich regions. Our results imply that there is no need to suppose the existence of a fourth NF-Y subunit in higher eukaryotes (15). Recapitulation of NF-Y activation with GAL4 constructs allows us to now tackle the questions concerning the target(s) of NF-Y in the general transcription machinery by functional *in vivo* experiments.

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